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Quantitative Determination of Oestradiol-17β Hydroxysteroid Dehydrogenase: Increased Sensitivity by HPLC Separation of the Hormones Permits the Measurement of Enzyme Activity in Cryostat Sections[1], [2]

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Summary: The activity of the oestradiol-17β hydroxysteroid dehydrogenase in human endometrial and breast cancer specimens was determined by the NAD-dependent conversion of oestradiol-17β to oestrone. The sensitivity of the determination was improved by the separation of the hormones by HPLC. We are now able to determine oestradiol-17β hydroxysteroid dehydrogenase quantitatively in cryostat sections. A clear correlation of serum progesterone levels and oestradiol-17β hydroxysteroid dehydrogenase activity in the endometrium was demonstrated, and we found a more than 30-fold increase in enzyme activity after the progesterone surge. In contrast, in breast cancer samples, we found no correlation between oestradiol-17β hydroxysteroid dehydrogenase and the measured serum parameters.

Introduction

The oestradiol-17β hydroxysteroid dehydrogenase in the human endometrium is thought to be induced directly by progesterone during the menstrual cycle and, therefore, is a tool in assessing progesterone action. Additionally, oestradiol-17β hydroxysteroid dehydrogenase is suggested as a parameter in the diagnosis of breast cancer by a variety of authors (1–6).

A highly accurate method was required for the determination of very low oestradiol-17β hydroxysteroid dehydrogenase activities in minimal quantities of biopsy tissue or in tissues such as breast cancer specimens, which contain low levels of enzyme activity. This would enable the investigation of possible correlations between cytosolic oestrogen and progesterone receptors and oestradiol-17β hydroxysteroid dehydrogenase activity. To develop such a method, it was firstly necessary to minimize tailings of radioactive oestradiol during the chromatographic separation steps, and secondly to handle small quantities of tissue.

Like other investigators we measured oestradiol-17β hydroxysteroid dehydrogenase activity by the NAD/NADH-dependent conversion of tritiated oestradiol to oestrone. However, we introduced HPLC for the isolation of the reaction products, oestradiol and oestrone, whereas previous authors have used thin-layer chromatography (1, 4, 7–9), paper chromatography (10), hydroxyalkoxypropyl Sephadex microcolumns (11) or lipidex microcolumns (12) for this purpose.

We approached the second problem, the determination of cytosolic receptors and oestradiol-17β hydroxysteroid dehydrogenase in parallel, by determin-

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[2]) Enzymes:
- Oestradiol-17β hydroxysteroid dehydrogenase; (Hydroxysteroid-17β:NAD$^+$-oxidoreductase (EC 1.1.1.63)).
- Glutamate dehydrogenase (EC 1.4.1.3.)
- Alkaline phosphatase (EC 3.1.3.1.)

ing oestradiol-17β hydroxysteroid dehydrogenase activity either in tissue homogenates prior to ultracentrifugation, or in cryostat sections of the tissue.

In the present work, we describe the use of HPLC in the oestradiol-17β hydroxysteroid dehydrogenase assay procedure. The method was checked against a standard assay in the widely examined human uterine system (7–9, 13–15). We also describe the application of this procedure to cryostat-sectioned material and compare it with our standard assay procedure. The results are relevant to the determination of oestradiol-17β hydroxysteroid dehydrogenase in the diagnosis of breast cancer.

Materials and Methods

Tissue collection and storage

Human endometrial tissue was obtained by curretage or after hysterectomy. Human breast cancer carcinomas were obtained immediately after surgical removal. Tissue samples freed of fat and necrotic tissue were stored in liquid nitrogen.

Preparation and incubation of tissue homogenates

After snap freezing in liquid nitrogen the tissue was ground to powder by three passages through a microdismembrator (Braun, F. R. G.) with intermediate cooling in liquid nitrogen. For oestradiol-17β hydroxysteroid dehydrogenase determination the tissue powder was diluted with 5 or 10 volumes (mg + µl) of buffer (5 mmol/l Na_2HPO_4, 1 mmol/l dithiothreitol, 10 mmol/l $Na_2MoO_4 \cdot 2H_2O$, and 115 ml/l glycerol (87%); (pH = 8.5). If oestrogen and gestagen receptors were to be determined as well, the dilution was performed with EORTC-buffer (10 mmol/l NaH_2PO_4, 1.5 mmol/l EDTA, 10 mmol/l $Na_2MoO_4 \cdot 2H_2O$, 115 ml/l glycerol (87%) and 1 ml/l monothioglycerol; pH = 7.4). Enzyme activity was determined either directly from the crude homogenate or after preparation of the cytosol by ultracentrifugation from the high speed pellet (144 000 *g*) resuspended in buffer.

The oestradiol-17β hydroxysteroid dehydrogenase assay was adapted from methods of *Tseng & Gurpide* (7), *Pollow* et al. (8) and *King* et al. (9) and always performed in duplicate or triplicate, respectively. In brief, 6.5 nmol of oestradiol (Merck, F. R. G.) in ethanol and 0.5 µCi = 18.5 kBq [^{3}H]oestradiol (40–60 Ci/mmol = 1.5 – 2.2 TBq/mmol, NEN, Dreieichenhain) were evaporated to dryness and redissolved in 250 µl buffer to give a final assay concentration of 20 µmol/l. Then 25 µl homogenate or buffer as a control were added and the reaction was started by the addition of 50 µl NAD (2 g/l). After 15–60 min of incubation at 37 °C, 10 µmol each of oestradiol and oestrone were added, prior to the addition of 2.5 ml ether/chloroform (3 + 1, by vol.) and extraction. The aqueous phase was reextracted with another 2.5 ml ether/chloroform and the organic phase was evaporated to dryness and redissolved in 100 µl acetonitrile : water : glacial acetic acid (38 + 60 + 2, by vol.) for HPLC.

For quantitation, the oestrogens were separated by a reversed phase HPLC-technique on a 5 µm ODS-Hypersil column (125 × 4.6 mm; Shandon, England) with the eluent described above. The loss during extraction was estimated directly from the oestrone added as carrier, by UV-detection or by the addition of a [^{14}C]oestradiol (NEN, Dreieichenhain) standard prior to extraction.

Protein was determined according to the method of *Bradford* (16) with human serum albumin as a standard. Prior to determination, homogenates or resuspended pellets were solubilized in the presence of 0.3 mol/l KOH for 20 min at 50 °C.

Preparation and incubation of cryostate sections

Duplicate sets of cryostat sections (30 µm) to give a final section area of about 1 cm^2 were collected on small glass coverslips and gently crushed into glass tubes containing 275 µl buffer supplemented with 20 µmol/l of oestradiol and with 0.5 µCi = 18.5 kBq tritiated oestradiol (40–60 Ci/mmol = 1.5 – 2.2 TBq/mmol, NEN, Dreieichenhain). Incubation, extraction and separation were performed as described above for the standard assay. For protein determination the small remaining organic phase was evaporated and the complete residue solubilized in the presence of 0.3 mol/l KOH.

Results

Oestradiol-17β hydroxysteroid dehydrogenase activity in homogenates of the endometrium

In order to establish an assay for oestradiol-17β hydroxysteroid dehydrogenase which allows the measurement of either very low enzyme activities or enzymatic activities in a minimal amount of tissue, it was necessary to minimize contamination and tailing during the separation of the enzymatically formed oestrone. In the reversed phase HPLC-separation described here, the tailing of oestradiol is less than 1‰. The mean deviations in the HPLC-assay of duplicate determinations in endometrial tissue depend on the activity of the sample. We found deviations up to 10% for activities < 1 nmol/mg × h (0.024–0.54 nmol/mg × h; mainly proliferative tissue) and below 5% for activities > 1 nmol/mg × h (1.88–9.1 nmol/mg × h; only secretory samples).

We characterized the enzyme kinetically in homogenates and found a saturating concentration of oestradiol of 20 µmol/l and an apparent K_m = 2 µmol/l in a *Hanes* (17) linearisation of the *Michaelis-Menten* equation. Oestradiol-17β hydroxysteroid dehydrogenase catalytic activity increases linearly with increasing protein concentration, i. e. the specific activity is independent of the protein concentration in the test up to a fourfold dilution.

Endometrial oestradiol-17β hydroxysteroid dehydrogenase activity during the menstrual cycle

Oestradiol-17β hydroxysteroid dehydrogenase activity appears in significant amounts during the second half of the menstrual cycle after the progesterone surge. We examined 26 individual samples in more

than 70 duplicate determinations and found a good correlation of the serum progesterone concentration and the oestradiol-17β hydroxysteroid dehydrogenase activity in the endometrium (fig. 1 b, r = 0.94). It is worth mentioning that ten tissue samples which were affected by endometriosis also showed a correlation of serum progesterone concentrations and oestradiol-17β hydroxysteroid dehydrogenase activity (data not shown).

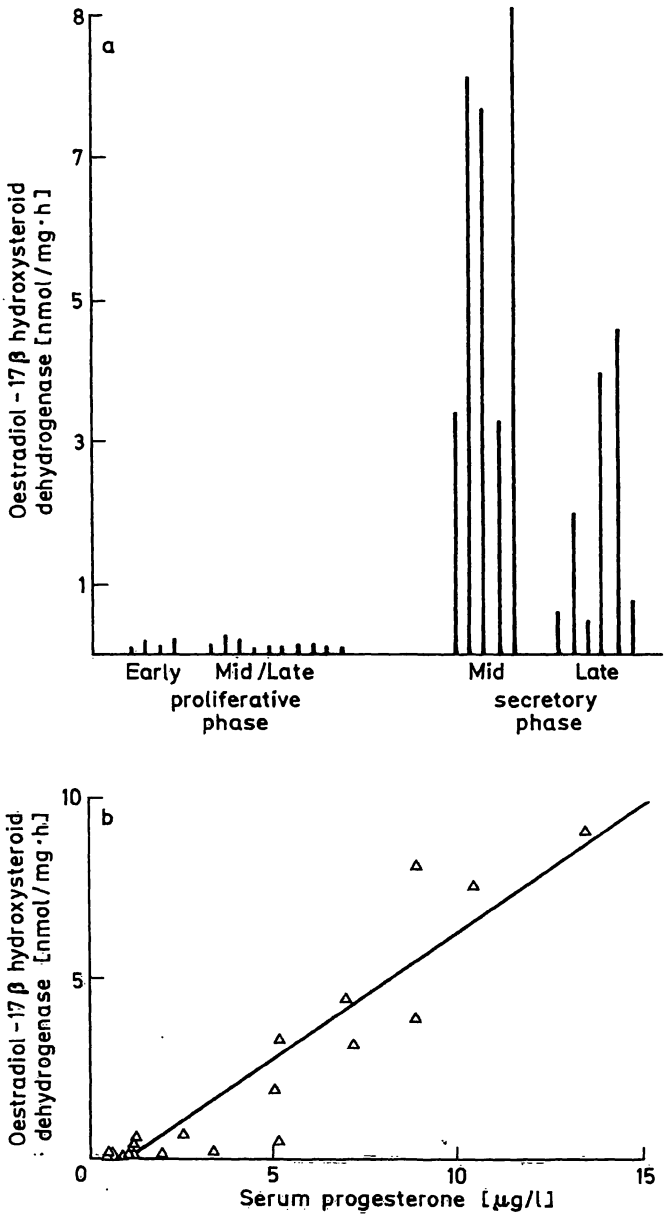

Fig. 1. Oestradiol-17β hydroxysteroid dehydrogenase during the menstrual cycle:
in a) oestradiol-17β hydroxysteroid dehydrogenase activities (nmol/mg × h) of individual samples (each line represents an individual patient) are plotted according to the respective phase of the menstrual cycle;
b) clearly demonstrates that oestradiol-17β hydroxysteroid dehydrogenase levels (nmol/mg × h) in the endometrium correlate well (r = 0.94, 26 patients 72 individual pieces of tissue) with serum progesterone (µg/l) levels.

A significant rise in enzymatic activity from the proliferative to the secretory phase of the menstrual cycle was apparent, although there is a considerable scatter of the enzymatic activity from one sample to the other (fig. 1 a). Compared with other authors we found considerably lower enzymatic activities during the proliferative phase, so that we noted a thirtyfold increase in enzyme activity from that of the proliferative endometrium (0.18 ± 0.17 nmol/mg × h) to the highest activity measured in the mid luteal endometrium (6.26 ± 2.8 nmol/mg × h).

Determination of oestradiol-17β hydroxysteroid dehydrogenase in homogenates of separate pieces of tissue of the same uterus without a parallel histological examination can lead to scattered results, which are unsuitable for interpretation. For explanation see table 1. Each sample consisted of four or five independent pieces of tissue. After the examination of individual pieces of tissue of either patient the results from sample 1 appear to be rather homogeneous while sample 2 is rather heterogeneous with respect to oestradiol-17β hydroxysteroid dehydrogenase activity. For comparison we add the results obtained with piece 5 of the second sample in the cryostat assay.

To avoid the difficulties exemplified in table 1 it is necessary to relate biochemical determinations to histological controls. This can be achieved if oestradiol-17β hydroxysteroid dehydrogenase is determined quantitatively in cryostat sections, including a histological examination of parallel sections.

Tab. 1. Oestradiol-17β hydroxysteroid dehydrogenase activity in homogenates of individual samples

Sample	7254 nmol/mg × h	8023 nmol/mg × h
Piece 1	8.05	3.84
Piece 2	7.74	6.86
Piece 3	9.1	9.88
Piece 4	–	10.52
Piece 5	–	2.56*
Mean	8.3	6.73
Piece 5 sections	–	7.36*

* measured in the same piece of tissue

Oestradiol-17β hydroxysteroid dehydrogenase activity assay in cryostat-sectioned samples

To examine and to characterize tissue samples as completely as possible it is necessary to perform quantitative biochemical determinations and histology in parallel in a single piece of tissue. To validate quantitative oestradiol-17β hydroxysteroid dehydrogenase

determinations in cryostat-sectioned material we measured the protein content and enzyme activity in dependence on increasing numbers of cryostat sections. We always found a linear correlation of $r > 0.9$ for both protein and enzyme activity, dependent on the number of sections (fig. 2). A representative diagram is shown in fig. 2; the apparent K_m = 4 µmol/l (fig. 3). The saturating concentrations of oestradiol are about 20 µmol/l and, therefore, nearly identical in both types of experiment.

The specific oestradiol-17β hydroxysteroid dehydrogenase activity in cryostat sections is 1.3 – 5.5 times greater than the activities measured in homogenates. These comparative studies which are discussed below were performed on individual pieces of tissue cut into two halves.

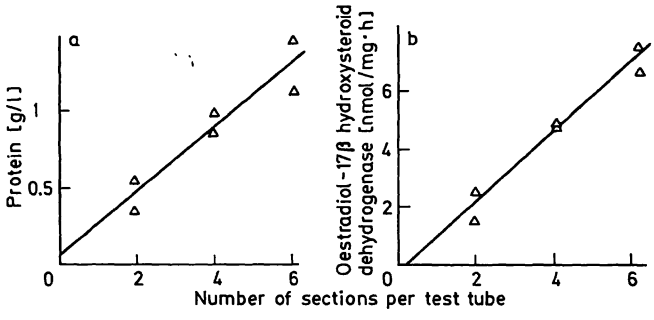

Fig. 2. Oestradiol-17β hydroxysteroid dehydrogenase activity in tissue sections:
here the linear relationship of a) protein content (mg/l), b) oestradiol-17β hydroxysteroid dehydrogenase and the number of sections per test tube is demonstrated.

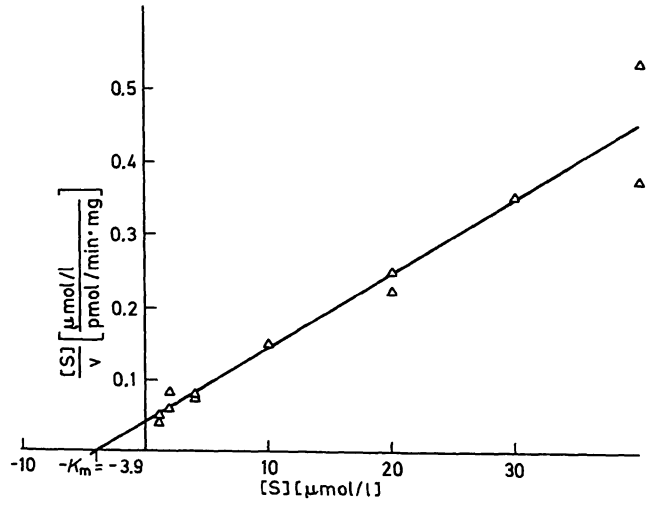

Fig. 3. K_m of oestradiol-17β hydroxysteroid dehydrogenase determined in sections:
The mean K_m (approx. 4 µmol/l) estimated with the section method by the *Hanes* linearization of the *Michaelis-Menton*-equation is higher than that obtained after incubation of homogenates (2 µmol/l), but it is of comparable magnitude.

The limiting factor in determining oestradiol-17β hydroxysteroid dehydrogenase activity by the combination of cryostat sectioning of the tissue and HPLC-separation of the reaction products is not the enzymatic activity itself but the reliable determination of the protein content. Thus, in a secretory tissue sample we were able to determine oestradiol-17β hydroxysteroid dehydrogenase activity in sections of 10 – 30 µm thickness, but we needed two sections of 30 µm thickness of the same specimen covering about 0.5 cm^2 area to get a reliable and reproducible protein determination.

Oestradiol-17β hydroxysteroid dehydrogenase in breast cancer tissue

In addition, our assay procedure should also provide a suitable tool for the determination of relatively low oestradiol-17β hydroxysteroid dehydrogenase activities. To prove this hypothesis we chose breast cancer tissue which, if compared with endometrial tissue, is expected to contain relatively low enzymatic activities. We examined 54 tumour samples for oestradiol-17β hydroxysteroid dehydrogenase, and found activities ranging from 0 – 32 nmol/mg × h. The mean activity of 30 tumour samples showing no peculiarities in the histological examination was 0.21 ± 0.16 nmol/mg × h. As a control, no significant activities of oestradiol-17β hydroxysteroid dehydrogenase were detected in cytosols of the tumour tissue.

We were not able to correlate oestradiol-17β hydroxysteroid dehydrogenase activity with progesterone- and oestrogen receptor levels in the tissue or with lutropin, follitropin, progesterone and oestradiol content in the serum. However, all samples with an increased level of oestradiol-17β hydroxysteroid dehydrogenase activity possess some peculiarities in their histology. But this finding is not of diagnostic value, because even more tissue samples exist with the same peculiarities but without increased oestradiol-17β hydroxysteroid dehydrogenase activities.

Discussion

HPLC separation

The aim and scope of our study was to establish an enzyme assay for either low activities of oestradiol-17β hydroxysteroid dehydrogenase or for activities in minimal amounts of tissue like cryostat sections or biopsies; this would enable the determination of en-

zyme activity in parallel with the biochemical and/or immunological determinations of oestrogen and progesterone receptors by concomitant histological examination. We therefore introduced HPLC to minimize the tailing of oestradiol during the separation procedure, which would otherwise interfere with the determination of oestrone.

One problem was the internal standardisation of the extraction procedure. To overcome this difficulty, losses during the extraction procedure were monitored twice, either by the UV-detection of the unlabelled oestrone which was added as carrier and comparison with a standard curve, or by determining the loss of a [^{14}C]oestradiol standard. Both procedures have disadvantages. In samples with low oestradiol-17β hydroxysteroid dehydrogenase activity, enzymatically formed oestrone can be neglected in comparison with the large excess of oestrone added as carrier. This does not apply for activities > about 5 nmol/mg × h, when enzymatically formed oestrone interferes with the determination of the carrier oestrone. On the other hand, the addition of a [^{14}C]oestradiol standard produces the well-known difficulties of double isotope techniques. However, the total amount of tritiated oestradiol must be determined, to avoid the necessity of indirect measurement. We suggest that the [^{14}C]standard should be omitted whenever possible. In our view it is better to shorten the incubation period if a crude estimate of the expected activity is available.

Oestradiol-17β hydroxysteroid dehydrogenase in endometrial homogenates

We checked our assay system with human endometrial tissue and compared our data with those of *Tseng* and *Gurpide* (7, 13) and *Pollow* et al. (8, 14). As described above, our data for the luteal endometrium are comparable to the findings of these authors. But we found significantly lower activities during the proliferative phase of the menstrual cycle. According to our results there is a more than thirty-fold increase in enzyme activity after the progesterone surge. This is about three-fold higher than described in the literature (citations see above). We feel that these findings are probably due to diminished background activities.

Limitations and advantages of the cryostat section method

We found no general limitations in the determination of the oestradiol-17β hydroxysteroid dehydrogenase activity in cryostat sections. As stated above, a greater limitation is the accurate determination of the protein content. However, we found some interference with the determination of the enzyme activity when diffusion was hindered by the overlap of two or more sections. For this reason, sections must be arranged carefully side by side when performing this assay.

Despite these limitations, preparation of tissue by sectioning seems to be a very gentle procedure, as can be deduced from the finding of about three-fold higher enzyme activities in sections. This assumption is further substantiated by the fact that glutamate dehydrogenase, a mitochondrial enzyme, is increased two-fold and alkaline phosphatase, a microsomal enzyme, is enriched by a factor of 1.2; oestradiol-17β hydroxysteroid dehydrogenase is located predominately in these two subcellular fractions (8). A great advantage of the assay is that it provides the opportunity to correct biochemical data directly according to histological staging.

Oestradiol-17β hydroxysteroid dehydrogenase in the diagnosis of breast cancer

We feel that oestradiol-17β hydroxysteroid dehydrogenase is only of very limited use in breast cancer diagnosis, because we were not able to correlate oestradiol-17β hydroxysteroid dehydrogenase activity to any other parameter measured. such as cytosolic oestrogen and progesterone receptors, or concentrations of lutropin, follitropin, oestradiol or progesterone in the serum. Obviously, there is no correlation with the age of the patient. However, enriched concentrations of oestradiol-17β hydroxysteroid dehydrogenase might provide evidence for accompanying anomalies, but a specific kind of malignancy cannot be defined by assaying oestradiol-17β hydroxysteroid dehydrogenase. The high oestradiol-17β hydroxysteroid dehydrogenase levels which occasionally occur might in part be explained by the findings of *McNeill* et al. (18) who demonstrated that the epidermal growth factor, the transforming growth factor and homogenates of breast cancer tissue can stimulate oestradiol-17β hydroxysteroid dehydrogenase in adipose tissue.

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References

1. Bonney, R. C., Reed, M. J., Davidson, K., Beranek, P. A. & James, V. H. T. (1983) Clin. Endocrinol. *19*, 727–739.
2. Beranek, P. A., Folkered, E. J., Ghilchick, M. W. & James, V. H. T. (1984) Clin. Endocrinol. *20*, 205–212.
3. Beranek, P. A., Folkered, E. J., Newton, C. J., Reed, M. J., Ghilchick, M. W. & James, V. H. T. (1985) Int. J. Cancer *36*, 685–687.
4. Fournier, S., Kuttenn, F., DeCicco, F., Baudot, N., Malet, C. & Mauvais-Jarvis, P. (1982) J. Clin. Endocrinol. Metab. *55*, 428–433.
5. Fournier, S., Brihmat, F., Durand, J. C., Sterkers, N., Martin, P. M., Kuttenn, F. & Mauvais-Jarvis, P. (1985) Cancer Res. *45*, 2895–2899.
6. Satyaswaroop, P. G. & Mortel, R. (1982) Cancer Res. *42*, 1322–1325.
7. Tseng, L. & Gurpide, E. (1974) Endocrinology *94*, 419–423.
8. Pollow, K., Lübbert, H., Boquoi, E., Kreutzer, G., Jeske, R. & Pollow, B. (1975) Acta Endocrinol. *79*, 134–145.
9. King, R. J. B., Whitehead, M. F., Campbell, S. & Minardi, J. (1978) Postgraduate Med. J. *54* (Suppl. 2), 65–68.
10. Folkered, E. J. & James, V. H. T. (1982) J. Steroid Biochem. *16*, 539–543.
11. Neumannova, M., Kauppila, A. & Vihko, R. (1983) Obstetr. Gynecol. *61*, 181–188.
12. Vierkko, P., Kauppila, A., Rönnberg, L. & Vihko, R. (1985) Fertility and Sterility *43*, 218–224.
13. Tseng, L., Gusberg, S. B. & Gurpide, E. (1977) Ann. NY Acad. Sci. *286*, 190–198.
14. Pollow, K. & Boquoi, E. (1976) Z. Krebsforsch. *86*, 231–242.
15. King, R. J. B., Whitehead, M. L., Campbell, S. & Minardi, J. (1979) Cancer Res. *39*, 1094–1101.
16. Bradford, M. M. (1976) Anal. Biochem. *72*, 248–254.
17. Hanes, C. S. (1932) Biochem. J. *26*, 1406–1421.
18. McNeill, J. M., Reed, M. J., Beranek, P. A., Newton, C. J., McGhilchick, M. W. & James, V. H. T. (1986) Cancer Letters *31*, 213–219.

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